

Selective inhibition of NF- κ B activation prevents dopaminergic neuronal loss in a mouse model of Parkinson's disease

Anamitra Ghosh*, Avik Roy*, Xiaojuan Liu†, Jeffrey H. Kordower*, Elliott J. Mufson*, Dean M. Hartley*, Sankar Ghosh‡, R. Lee Mosley§, Howard E. Gendelman§, and Kalipada Pahan*†¶

*Department of Neurological Sciences, Rush University Medical Center, Chicago, IL 60612; †Section of Neuroscience, Department of Oral Biology, University of Nebraska Medical Center College of Dentistry, Lincoln, NE 68583; ‡Section of Immunobiology and Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, CT 06536; §Center for Neurovirology and Neurodegenerative Disorders and Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198

Edited by Eugene Johnson, Washington University School of Medicine, St. Louis, MO, and accepted by the Editorial Board October 2, 2007 (received for review May 24, 2007)

Parkinson's disease (PD) is the second most common neurodegenerative disorder. Despite intense investigations, no effective therapy is available to stop its onset or halt its progression. The present study evaluates the ability of peptide corresponding to the NF- κ B essential modifier-binding domain (NBD) of I κ B kinase α (IKK α) or IKK β to prevent nigrostriatal degeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD and establish a role for NF- κ B in human parkinsonism. First, we found that NF- κ B was activated within the substantia nigra pars compacta of PD patients and MPTP-intoxicated mice. However, i.p. injection of wild-type NBD peptide reduced nigral activation of NF- κ B, suppressed nigral microglial activation, protected both the nigrostriatal axis and neurotransmitters, and improved motor functions in MPTP-intoxicated mice. These findings were specific because mutated NBD peptide had no effect. We conclude that selective inhibition of NF- κ B activation by NBD peptide may be of therapeutic benefit for PD patients.

MPTP | NBD peptides | neurodegeneration

Parkinson's disease (PD) is second only to Alzheimer's disease as the most common and debilitating age-associated human neurodegenerative disorder. A host of environmental, genetic, and immune cues have been associated with the onset of this disease (1). Clinical symptoms of PD include tremor, bradykinesia, rigidity, and postural instability (2). Pathologically, it is characterized by gliosis and progressive degeneration of the dopaminergic neurons associated with the presence of intracytoplasmic inclusions (Lewy bodies) in the substantia nigra pars compacta (SNpc) (3). In postmortem PD brains, affected dopaminergic neurons display morphological characteristics of apoptosis, including cell shrinkage, chromatin condensation, and DNA fragmentation (3, 4).

Although the disease mechanisms that cause PD are poorly understood, recent studies strongly support the role of inflammation in nigrostriatal degeneration in this disease (3, 5). First, early intervention with nonsteroidal antiinflammatory drugs slows disease incidence. Second, significant microglial activation occurs in close proximity to damaged or dying dopaminergic neurons. Third, the concentration of NO₂⁻ (nitrite), a metabolite of NO, increases in the cerebrospinal fluid (CSF) of patients with PD compared with patients without dopaminergic dysfunction (6). It has been shown that many cells in the SNpc from postmortem PD samples express considerable amounts of inducible nitric oxide synthase (iNOS), whereas those from age-matched controls do not (7). Consistently, the ablation of iNOS in mutant mice significantly attenuates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity, thus indicating its role in MPTP-induced SNpc dopaminergic neurodegeneration (8). Fourth, a variety of proinflammatory cytokines including TNF- α , IL-1 β , IL-6, eicosanoids, and other immune neurotoxins are found in either CSF or affected brain

regions in PD (9). Indeed, concentrations of IL-1 β and IL-6 in SNpc and blood are significantly higher in PD than age-matched subjects without any neurological disease (10). Subsequently, it has been shown that the secretion of IL-1 β , IL-6, and TNF- α is significantly enhanced in peripheral blood mononuclear cells of PD patients compared with age-matched controls (11). Finally, transgenic mice carrying homozygous mutant alleles for both the TNF receptors, but not the individual receptors, are completely protected against MPTP intoxication (12). Taken together, these studies demonstrate the importance of inflammation for disease manifestation and for developing treatment paradigms.

Promoter regions of proinflammatory molecules contain the DNA binding site for NF- κ B (13). Furthermore, the inhibition of NF- κ B activation reduces the induction of proinflammatory molecules (13). These results strongly suggest that NF- κ B is an important regulator of inflammation. Activation of NF- κ B requires the activity of I κ B kinase (IKK) complex containing IKK α and IKK β and the regulatory protein NF- κ B essential modifier (NEMO) (13). Ghosh and colleagues (14) have shown that peptides corresponding to the NEMO-binding domain (NBD) of IKK α or IKK β specifically inhibit the induction of NF- κ B activation without inhibiting basal NF- κ B activity. Here, we demonstrate that NF- κ B activation is induced *in vivo* in the SNpc of PD patients and MPTP-intoxicated mice. Interestingly, treatment of MPTP-intoxicated mice with NBD peptide results in significant protection of nigrostriatal neurons against MPTP-induced neurodegeneration.

Results

NBD Peptide Inhibits MPP⁺-Induced Glial NF- κ B Activation. Glial inflammation is a critical component of PD pathogenesis (3–5, 15), which is mirrored in the MPTP mouse model. The neurotoxic effect of MPTP depends on its conversion into MPP⁺. In glial cells, monoamine oxidase B converts MPTP to MPP⁺, which then leads to glial activation (2, 3). Therefore, we first investigated whether MPP⁺ could activate proinflammatory transcription factor NF- κ B in astrocytes and microglia and whether NBD peptides suppressed its activity. Activation of NF- κ B was monitored by both DNA-binding and transcriptional activities (16, 17). MPP⁺ alone induced

Author contributions: A.G., A.R., and K.P. designed research; A.G., A.R., X.L., and R.L.M. performed research; J.H.K., E.J.M., D.M.H., S.G., R.L.M., and H.E.G. contributed new reagents/analytic tools; A.G., A.R., J.H.K., E.J.M., D.M.H., S.G., R.L.M., H.E.G., and K.P. analyzed data; and A.G., J.H.K., E.J.M., D.M.H., S.G., H.E.G., and K.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. E.J. is a guest editor invited by the Editorial Board.

¶To whom correspondence should be addressed at: Department of Neurological Sciences, Rush University Medical Center, 1735 West Harrison Street, Suite 320, Chicago, IL 60612. E-mail: kalipada.pahan@rush.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0704908104/DC1.

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the activation of NF- κ B in primary human astrocytes as evidenced by DNA-binding [supporting information (SI) Fig. 6A Upper] and transcriptional activities (SI Fig. 6A Lower). However, wild-type NBD (wtNBD) but not mutated NBD (mNBD) peptides inhibited MPP⁺-induced activation of NF- κ B in primary human astrocytes in a dose-dependent manner (SI Fig. 6A). Similarly, MPP⁺ also induced the activation of NF- κ B in mouse BV-2 microglial cells, and wtNBD but not mNBD peptides suppressed MPP⁺-induced DNA-binding (SI Fig. 6B Upper) and transcriptional activities (SI Fig. 6B Lower) of NF- κ B. These results demonstrate that NBD peptides are capable of inhibiting MPP⁺-induced activation of NF- κ B in astrocytes and microglia.

Activation of NF- κ B in MPTP-Intoxicated Mice and in PD Patients. To investigate the role of induced activation of NF- κ B in the loss of invaluable dopaminergic neurons in MPTP-intoxicated mice, we examined whether the activation of NF- κ B was induced in mid-brains of affected mice. MPTP intoxication led to robust expression of RelA p65 in the SNpc compared with saline treatment (Fig. 1B). Double-label immunofluorescence analysis indicates that p65 was principally expressed by microglia and astrocytes. This p65 was present in both nucleus and cytoplasm because part of it did not colocalize with DAPI (Fig. 1B). To further monitor the induced activity of NF- κ B, we performed EMSA on nuclear extracts isolated from ventral midbrain tissues of saline- and MPTP-treated mice. Consistent with an increase in p65 expression, MPTP intoxication markedly induced the DNA-binding activity of NF- κ B in ventral midbrain (Fig. 1C). These results show that parkinsonian neurotoxin MPTP is capable of inducing the activation of NF- κ B within astroglia and microglia *in vivo* in the midbrain. Next, we wondered whether activation of NF- κ B also takes place within astroglia and microglia *in vivo* in the midbrain of PD patients. As revealed from immunofluorescence analysis of midbrain sections, the expression of p65 protein was greater in substantia nigra (SN) of PD brain compared with age-matched controls (Fig. 1D). Earlier, Hunot et al. (18) demonstrated increased nuclear translocation of NF- κ B p65 in dopaminergic neurons of patients with PD. By double-labeling experiments, we also detected p65 within tyrosine hydroxylase (TH)-positive neurons in PD midbrain tissues (data not shown). In addition, a marked colocalization of p65 with GFAP-positive activated astroglia and CD11b-positive activated microglia was observed in SN of PD patients (Fig. 1D). This increased p65 was also present in both nucleus and cytoplasm (Fig. 1D). These results suggest that, in addition to neurons, NF- κ B is also activated within microglia and astroglia, cells that produce proinflammatory molecules in the CNS of patients with neurodegenerative disorders including PD.

NBD Peptide Inhibits the Induction of NF- κ B Activation *in Vivo* in the Midbrain of MPTP-Intoxicated Mice. As evident from SI Fig. 6, cell-permeable NBD peptides capable of blocking NEMO/IKK interactions inhibit MPP⁺-induced activation of NF- κ B in astrocytes and microglia. However, before testing clinical efficacy of NBD peptides in the MPTP mouse model of PD, it was important to determine whether these peptides entered into the CNS and were capable of inhibiting the induction of NF- κ B activation *in vivo* in the midbrain of MPTP-intoxicated mice. We quantified the level of wtNBD peptide (101.5 ± 34 ng/mg of tissue in comparison with nil in control mice) by HPLC in brain of mice after 12 h of i.p. injection (0.75 mg/kg body weight). After tail vein injection, we also detected this peptide in different parts of the brain by infrared scanning (SI Fig. 7). These results demonstrate that NBD peptide can ingress into the brain. The treatment schedule of mice with this peptide is shown in Fig. 1A. As seen in Fig. 1B and C, wtNBD but not mNBD peptides inhibited the expression of p65 and suppressed the DNA-binding activity of NF- κ B *in vivo* in the midbrain of MPTP-intoxicated mice. Moreover, no significant inhibition of p65 was

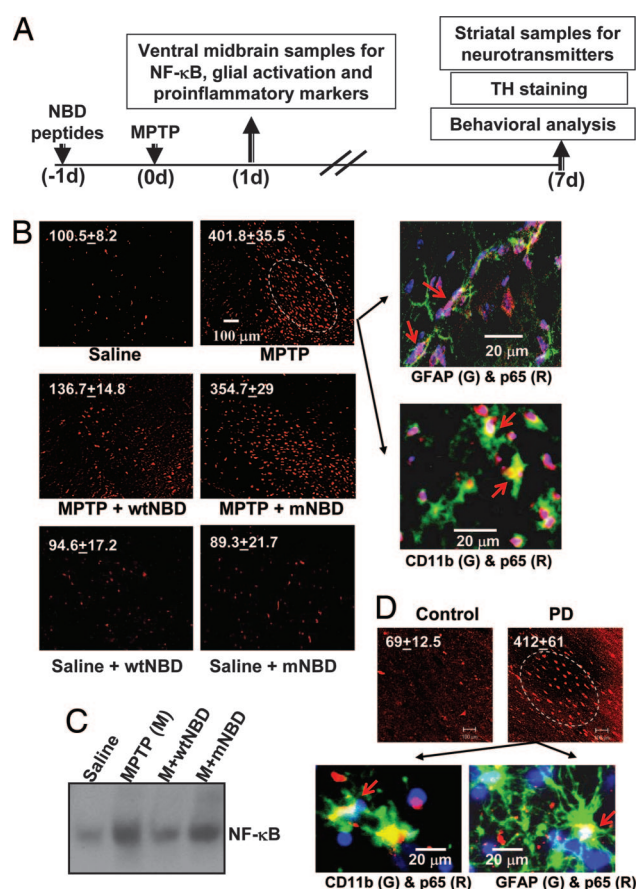


Fig. 1. Activation of NF- κ B in ventral midbrain of MPTP-intoxicated mice and PD patients. (A) Treatment schedule of MPTP-intoxicated mice with NBD peptide. (B) Twenty-four hours after the last injection of MPTP, ventral midbrain sections were immunostained for p65. (Magnification, $\times 10$.) NF- κ B p65-positive cells counted in four nigral sections (two images per slide) of each of four mice in an Olympus IX81 fluorescence microscope using the MicroSuite imaging software are mentioned as cells per square millimeter at the upper left corner of each image. Midbrain sections of MPTP-intoxicated mice were also double-labeled for p65 and glial cell markers (GFAP for astrocytes and CD11b for microglia). (Magnification, $\times 60$.) DAPI was used to visualize nucleus. (C) EMSA was carried out with nuclear extracts prepared from ventral midbrain tissues. (D) Midbrain sections of PD patients and age-matched controls were immunostained for p65. (Magnification, $\times 10$.) Number of p65-positive cells (cells per square millimeter) is mentioned at the upper left corner of each image. Midbrain sections of PD patients were also double-labeled for p65 and glial cell markers (GFAP and CD11b). (Magnification, $\times 60$.) The arrows indicate colocalization of p65 with DAPI. Results represent analysis of four different sections from each of four different brains.

seen by either wtNBD or mNBD peptide in the midbrain of saline-treated mice (Fig. 1B).

NBD Peptide Inhibits Inflammation *in Vivo* in the Midbrain of MPTP-Intoxicated Mice. Inflammation plays a role in the loss of dopaminergic neurons in PD and its animal model (7–12). Because NBD peptides inhibited the activation of NF- κ B *in vivo* in the midbrain of MPTP-intoxicated mice, we examined whether these peptides were able to suppress the expression of various proinflammatory molecules in the midbrain. As shown by semiquantitative RT-PCR (Fig. 2A) and quantitative real-time PCR (Fig. 2B) experiments, MPTP intoxication led to marked increase in mRNA expression of iNOS, IL-1 β , and TNF- α in the midbrain. However, wtNBD, but not mNBD peptide, strongly inhibited MPTP-induced expression of these proinflammatory molecules *in vivo* in the midbrain (Fig. 2A and B). Immunofluorescence analysis for iNOS also shows that

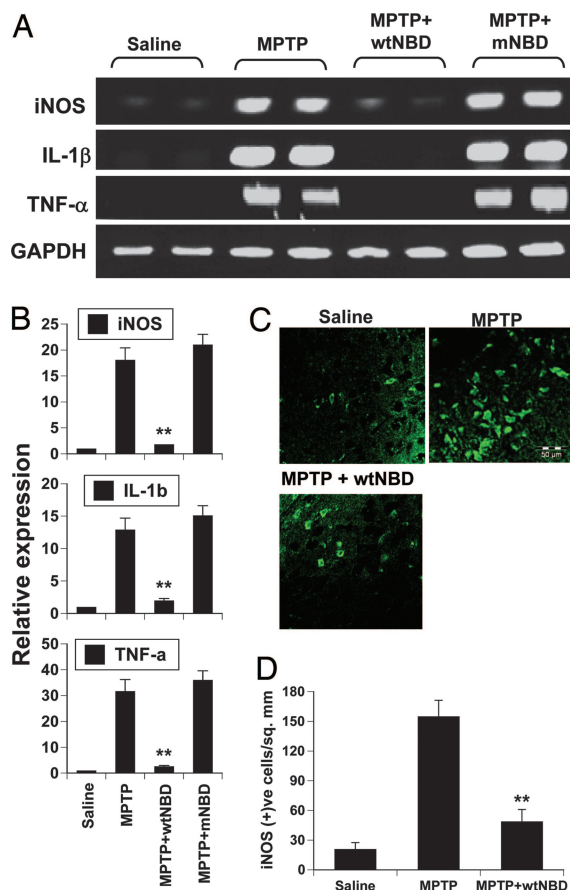


Fig. 2. NBD peptide inhibits the expression of proinflammatory molecules in ventral midbrain of MPTP-intoxicated mice. Mice receiving wtNBD and mNBD peptide (0.75 mg/kg body weight per day) from 1 day before MPTP insult were killed 24 h after the last injection of MPTP. The mRNA expression of iNOS, IL-1 β , and TNF- α was analyzed by RT-PCR (A) and quantitative real-time PCR (B). Data are means \pm SEM of five to six mice per group. (C) Ventral midbrain sections were immunolabeled for iNOS. (D) Cells positive for iNOS were counted in four nigral sections (two images per slide) of each of four mice as described above. **, $P < 0.001$ vs. the MPTP group.

MPTP intoxication led to marked increase in nigral iNOS protein expression and that wtNBD peptides suppressed MPTP-induced expression of iNOS protein (Fig. 2 C and D).

Recently, glial activation is being considered as a pathological hallmark in PD and other neurodegenerative disorders (3, 5, 15, 19). We investigated whether NBD peptides were capable of attenuating MPTP-induced activation of glial cells. Increased expression of CD11b, the β -integrin marker of microglia, represents microglial activation during neurodegenerative inflammation (20). Similarly, on activation, astrocytes also express enhanced level of GFAP, which is considered as a marker protein for astrogliosis (21). MPTP intoxication led to marked increase in mRNA expression of both CD11b and GFAP in the midbrain (Fig. 3 A and B). However, similar to the inhibition of proinflammatory molecules, wtNBD but not mNBD peptides strongly suppressed MPTP-induced expression of CD11b and GFAP mRNAs *in vivo* in the midbrain (Fig. 3 A and B). As evident from immunofluorescence analysis of CD11b in ventral midbrain sections (Fig. 3 C and D), MPTP intoxication led to marked increase in nigral CD11b protein expression and wtNBD peptides suppressed MPTP-induced expression of CD11b.

NBD Peptide Protects the Nigrostriatum After MPTP Intoxication. Seven days after the last injection of MPTP, brains were processed for quantification of SNpc dopaminergic cell bodies and their

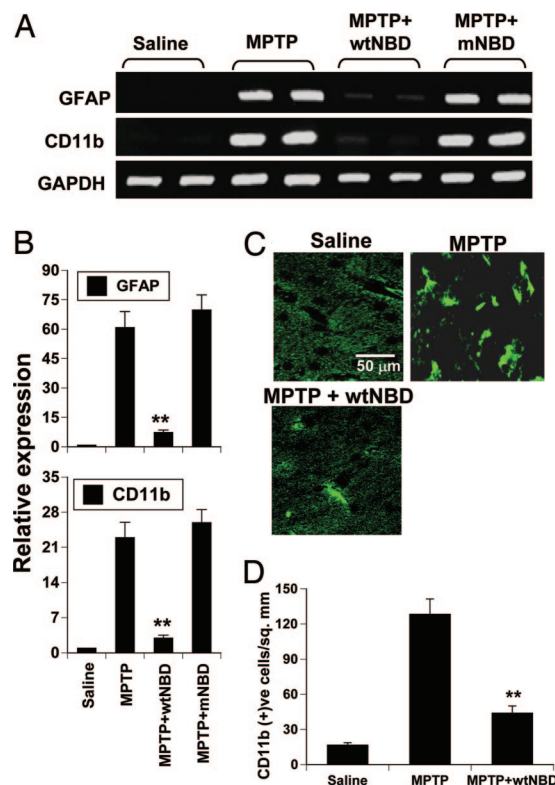


Fig. 3. NBD peptide inhibits the activation of glial cells in ventral midbrain of MPTP-intoxicated mice. Mice receiving wtNBD and mNBD peptides (0.75 mg/kg body weight per day) from 1 day before MPTP insult were killed 24 h after the last injection of MPTP. The mRNA expression of GFAP and CD11b was analyzed by RT-PCR (A) and quantitative real-time PCR (B). Data are means \pm SEM of five to six mice per group. (C) Ventral midbrain sections were immunolabeled for CD11b. (D) CD11b-positive cells were counted in four nigral sections (two images per slide) of each of four mice. **, $P < 0.001$ vs. the MPTP group.

projecting dopaminergic fibers in the striatum by TH immunostaining. MPTP intoxication led to a $\approx 68\%$ loss of SNpc TH-positive neurons (Fig. 4 A and B) and an 80% reduction of striatal TH ODs (Fig. 4 A and C) compared with saline-injected controls. However, in MPTP-intoxicated mice treated with wtNBD peptides, SNpc TH-positive neurons and striatal TH ODs were, in part, restored to near control levels (Fig. 4 A–C). In contrast, mNBD peptides failed to protect the nigrostriatal axis from MPTP intoxication.

Next, to determine whether NBD peptide protects against biochemical deficits caused by MPTP, we quantified levels of dopamine (DA) and two of its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), in striata 7 days after the MPTP treatment. MPTP intoxication led to $\approx 67\%$ decrease in striatal DA compared with striata of saline-injected mice (Fig. 4D). In contrast, MPTP-intoxicated animals that received wtNBD peptide showed only a 16% decrease in striatal dopamine (Fig. 4D). On the other hand, mNBD peptide had no such protective effect. Although wtNBD peptide exhibited beneficial effects against MPTP toxicity, we acknowledge that it could also elicit adverse effects in normal mice. To address this question, we examined the levels of neurotransmitters in saline-treated mice after 7 days of treatment with wtNBD and mNBD peptides and found no changes in DA, DOPAC, and HVA (Fig. 4D).

NBD Peptide Improves Locomotor Functions in MPTP-Intoxicated Mice. The ultimate goal of neuroprotective therapies is to decrease functional impairments. Therefore, we monitored locomotor and open-field activities. As previously reported (22), MPTP injection caused a marked decrease in rotorod performance

intoxicated mice. Seventh, in PD, treatment begins after a diagnosis is made. wtNBD peptide administered 2 days after initiation of the disease still suppressed the demise of TH-positive neurons and concomitant loss of neurotransmitters, suggesting that this peptide has the capability of attenuating disease progression. Furthermore, this pattern of findings indicates that the effect observed is the result of true neuroprotection and not an artificial one either blocking the conversion of MPTP to MPP⁺ or preventing the uptake of MPP⁺ into dopaminergic terminals. Because activation of NF- κ B is also induced within the SNpc of patients with PD (ref. 18; Fig. 1D), our results suggest that NBD peptide may slow down the loss of nigrostriatal neurons in patients with PD.

Being a highly lipophilic molecule, MPTP readily crosses the blood–brain barrier (BBB). Markey *et al.* (23) have shown that, after systemic administration, MPTP reaches the brain within minutes. Once in the brain, MPTP is converted to MPDP⁺ and MPP⁺. The latter is believed to be responsible for glial activation and neuronal death (3, 4). Park *et al.* (24) have shown that MPP⁺ does not induce the activation of NF- κ B in dopaminergic neurons. However, Hunot *et al.* (18) have demonstrated increased colocalization of NF- κ B p65 with melanized neurons in the mesencephalon of postmortem PD brains. We also observed marked activation of nigral NF- κ B and detected few TH-positive cells that colocalized with p65 in postmortem PD brains. In addition, we noticed marked colocalization of p65 with CD11b-positive microglia and GFAP-positive astrocytes *in vivo* in the SNpc of postmortem PD brains. The parkinsonian neurotoxin MPP⁺ was also capable of activating NF- κ B in isolated human astrocytes and mouse microglial cells. Although the upstream mechanisms are not known, MPP⁺-induced activation of NF- κ B in astrocytes and microglia was sensitive to NBD peptide, suggesting that MPP⁺ induces the activation of NF- κ B in glial cells via IKK-dependent pathway. Once NF- κ B is activated, in collaboration with other proinflammatory transcription factors, NF- κ B drives the transcription of several proinflammatory molecules including iNOS, TNF- α , and IL-1 β in both microglia and astroglia (25, 26), which have been shown to play an important role in the loss of dopaminergic neurons in MPTP-intoxicated mice and PD patients (8–12). Recently, we have demonstrated that, once NO is produced via NF- κ B-dependent pathway, it up-regulates the expression of GFAP in astroglia and the expression of CD11b in microglia through NF- κ B-independent but guanylate cyclase–cGMP–protein kinase G-dependent pathways (27, 28). Because glial production of proinflammatory molecules and gliosis are important features of PD pathology, it appears that NF- κ B activation regulates these pathological features either directly or indirectly and that NBD peptide protects dopaminergic neurons in MPTP-intoxicated mice via suppression of NF- κ B-dependent pathological steps.

Protective effects in animal models of PD have been obtained with various molecules including glial cell line-derived neurotrophic factor, neurturin, BDNF, TGF- β , and basic FGF (29, 30). However, clinical application of those molecules has been limited because of difficulties in delivery and side effects. These peptides do not readily diffuse across the BBB or ventricular lining and have limited or unstable bioavailability and some toxicity (31). Peptides usually do not enter into the CNS, and, therefore, sending peptides across the BBB is an important area of research. Earlier, we demonstrated anti-neuroinflammatory and protective effect of NBD peptide in experimental allergic encephalomyelitis, an animal model of multiple sclerosis (MS) (32). Here, we present evidence that this peptide enters into the CNS, reduces nigral activation of NF- κ B, suppresses nigral expression of proinflammatory molecules, and attenuates nigrostriatal degeneration in MPTP-intoxicated mice. Ghosh and colleagues (14) have used Antennapedia homeodomain to make NBD peptides cell permeable. However, our current results in the animal model of PD and our earlier results in the animal model of MS (32) suggest that Antennapedia homeodomain may be used to send peptides across the BBB.

At present, no effective therapy is available to halt the progression of PD. Administration of a dopamine agonist or levodopa has been the standard treatment for PD. However, it is often associated with a number of side effects and unsatisfactory outcomes. Therefore, understanding the mechanism of the disease process of PD and development of effective neuroprotective therapeutic approach to halt the disease progression are of paramount importance. The MPTP mouse model is particularly useful in testing new therapeutic intervention in PD. Here, we have demonstrated that wtNBD peptide suppressed the activation of NF- κ B in MPP⁺-stimulated glial cells as well as in SNpc of MPTP-intoxicated mice and protected the nigrostriatum from MPTP toxicity. In addition to its involvement in inflammation, activation of NF- κ B is also known to protect cells by up-regulating several antiapoptotic genes (33). Therefore, one might expect an adverse event where NBD peptide may augment the death of dopaminergic neurons by inhibiting neuronal induction of NF- κ B activation. However, under that scenario, wtNBD peptide should also inhibit the induction of NF- κ B activation in cells producing proinflammatory molecules resulting in an inhibition of proinflammatory molecule production within the midbrain. As a result, the inflammatory insult on dopaminergic neurons would be reduced. Then proinflammatory molecules would not induce the activation of NF- κ B in dopaminergic neurons, and, in turn, dopaminergic neurons would not need induced NF- κ B activation to survive in the noninflamed midbrain.

In summary, we have demonstrated that NBD peptide enters into the CNS, blocks the activation of NF- κ B in the SNpc, inhibits the expression of proinflammatory molecules and the activation glial cells in the midbrain, protects the loss of dopaminergic neurons, and improves the behavioral functions in MPTP-intoxicated mice. Our results suggest that these peptides may be used for therapeutic intervention in PD and other neurodegenerative disorders where inflammation within the CNS plays an important role in disease pathogenesis.

Materials and Methods

Animals and MPTP Intoxication. Six- to 8-week-old male C57BL/6 mice were purchased from Harlan. Animal maintenance and experimental procedures were in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee. For MPTP intoxication, mice received four i.p. injections of MPTP-HCl (18 mg/kg of free base; Sigma) in saline at 2-h intervals. Control animals received equivalent injections of saline.

Human Brain Tissue. Autopsy brain tissues from four male PD patients and four control subjects were obtained from the Rush PD Center Brain Bank. PD patients and control subjects did not differ significantly for their mean age at death (PD, 74 \pm 3 years; control, 79 \pm 18 years). The mean postmortem interval for PD and controls were 4.1 \pm 0.8 and 10.9 \pm 1.1 h, respectively.

Reagents. Rabbit and goat anti-NF- κ B p65 and goat anti-GFAP were purchased from Santa Cruz Biotechnology. Rat anti-mouse CD11b and mouse anti-human CD11b were purchased from Abcam and Serotec, respectively. Cy2- and Cy5-conjugated antibodies were obtained from Jackson ImmunoResearch Laboratories.

NBD Peptides and Their Use in MPTP Mice. NBD peptides were synthesized in EZBiolab. wtNBD and mNBD peptides contain the Antennapedia homeodomain (lowercase) and IKK β (uppercase) segments, and positions of W \rightarrow A mutations are underlined (14): wtNBD, drqikiwfnrnmkwwkLDWSWL; mNBD, drqikiwfnrnmk-wkLDASAL.

Groups of mice ($n = 6$) were treated with either wtNBD or mNBD peptides (0.75 mg/kg body weight per day) in saline through i.p. injection either 1 day before or after 2 days of the MPTP insult (Fig. 1A).

Isolation of Nuclear Extracts from Midbrain Tissues. Ventral midbrain was frozen immediately on dry ice, homogenized in buffer A [10 mM Hepes (pH 7.5), 10 mM KCl, 0.1 mM EGTA, 1.0 mM DTT, 1 mM PMSF, 10 μ g/ml each leupeptin, antipain, aprotinin, and pepstatin A], placed on wet ice for 10 min, and then treated with 1.0% Nonidet P-40. Nuclei were separated from the cytosolic proteins and lipids by multiple centrifugation at $20,000 \times g$ for 15 min. The extracts were then resuspended in buffer C [25% glycerol, 0.4 M NaCl, 20 mM Hepes (pH 7.5), 1.0 mM EGTA, 1.0 mM EDTA, 1.0 mM DTT, 1 mM PMSF, 10 μ g/ml each leupeptin, antipain, aprotinin, and pepstatin A] and briefly sonicated on ice. Finally, nuclear extracts obtained by centrifugation at $12,000 \times g$ for 10 min were used for the EMSA.

DNA-Binding Activity of NF- κ B. It was performed by EMSA as described in ref. 16, using radiolabeled oligonucleotides containing the consensus binding sequence (underlined) for NF- κ B (5'-C CTG CTG GGG GAA ATC CCT TCC CGC-3').

Transcriptional Activity of NF- κ B. It was performed as described in refs. 17 and 34, using pBIIX-Luc (an NF- κ B-dependent reporter construct) and pRL-TK (a plasmid encoding *Renilla* luciferase, used as transfection efficiency control).

RT-PCR Analysis. RT-PCR was carried out as described in refs. 27 and 28 by using an RT-PCR kit from Clontech.

Real-Time PCR Analysis. It was performed in the ABI Prism 7700 sequence detection system (Applied Biosystems) as described in refs. 27 and 28.

Immunohistochemistry. Mice were killed, and their brains were processed for TH and thionin staining (15). Total numbers of TH- and Nissl-stained neurons in SNpc were counted stereologically with the Stereo Investigator software (MicroBrightField) using an optical fractionator (15). Quantitation of striatal TH immunostaining was performed as previously described (15). Optical density measurements were obtained by digital image analysis (Scion). Striatal TH optical density reflected dopaminergic fiber innervation. For immunofluorescence staining on fresh frozen sections, rat anti-mouse CD11b (1:300), mouse anti-human CD11b (1:200), goat anti-mouse GFAP (1:300), rabbit anti-NF- κ B p65 (1:100), and goat anti-NF- κ B p65 (1:100) were used. The samples were mounted and

observed under a Bio-Rad MRC1024ES confocal laser-scanning microscope.

HPLC Analysis. Striatal tissues were sonicated in 0.2 M perchloric acid containing isoproterenol (internal standard) and resulting homogenates were centrifuged at $20,000 \times g$ for 15 min at 4°C. After pH adjusting and filtration, 10 μ l of supernatant was injected onto a Eicompak SC-3ODS column (HPLC-ECD System Eicom-HTEC-500 from JM Science) and analyzed as described in ref. 28.

For measuring MPP⁺, striata collected from mice after 3 h of the last injection of MPTP were homogenized in 0.2 M perchloric acid containing isoproterenol and processed as above. Supernatants were then analyzed in Waters 2695 separation module HPLC system using the Phenomenex Luna C18 separation column (250 \times 4.6 mm; 280-nm UV wavelength) with an isocratic gradient consisting of mobile phase A (18 m Ω water with 0.01 M H₃PO₄) and mobile phase B (acetonitrile with 0.01 M H₃PO₄) (1:4) at the flow rate of 0.15 ml/min. For measuring NBD peptide, whole brains collected from mice after 12 h of i.p. injection of NBD peptides (0.75 mg/kg) were homogenized in PBS containing protease inhibitor mixture (Sigma) and amyloid- β 16–20 (Bachem Bioscience) as internal standard. Homogenates were centrifuged and supernatants were passed through 100-kDa (Millipore microconYM 100) and 30-kDa (Millipore microconYM 30) protein separation filters, respectively. Flow-through samples were concentrated in a speed-vac and then analyzed in HPLC as described above.

Behavioral Analysis. Open-field assays for locomotor activity and rotarod tests for coordinated movement were performed. Locomotor activity was measured after 7 days of the last dose of MPTP in Digiscan Monitor (Omnitech Electronics). Before any insult or treatment, mice were trained for 1 h daily for 7 consecutive days in the open field and for 20 min daily for 7 consecutive days on rotarod at different speeds. The actual data were collected at 22 rpm. In the open-field experiment, various parameters, such as horizontal activity, total distance, number of movements, movement time, rest time, stereotypy counts, rearing, mean distance, mean time, center distance, and center time, were measured. In rotarod, the feet movement of the mice was observed at different speeds. To eliminate stress and fatigues, mice were given a 2-min rest interval.

We thank Drs. Yaping Chu and Muhammad Nadeem for their help in this study. This study was supported by the Michael J. Fox Foundation and National Institutes of Health Grants NS48923 and NS39940.

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